Disulfide Reactive with Sulfite in Native Serum Albumin*

I. M. Kolthoff, M. Matsuoka, B. H. Tan, and W. S. Shore

ABSTRACT: "Reactive disulfide" in native albumin is defined as the number of disulfide groups/mole of protein which react with sulfite at pH 6. The sulfhydryl liberated is titrated amperometrically at pH 2 with ethylmercuric chloride (EMC) or mercuric chloride. Reactive disulfide can also be determined by direct amperometric titration with silver nitrate, mercuric chloride, or EMC of sulfhydryl formed with sulfite at pH 9. The sum of sulfhydryl and "reactive disulfide" in three lots of commercial crystalline bovine serum albumin has been found equal or close to 1/mole of albumin.

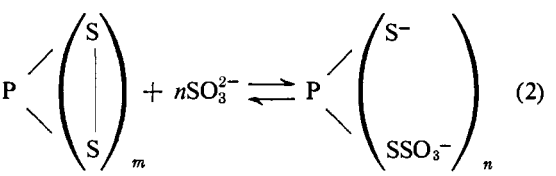
The sulfhydryl content of various lots of commercial crystalline bovine serum albumin (BSA)[1] has been found to vary between 0.45 and 0.80 SH/mole of BSA (Kolthoff *et al.*, 1965). King (1961) reported that albumin can contain cysteine and glutathione as a mixed disulfide

$$PSH + CySH \xrightarrow{\text{oxidation}} PSSCy \qquad (1)$$

where CySH refers to cysteine and PSSCy to the mixed disulfide in the protein. Hartley *et al.* (1962) were able to fractionate samples of BSA into mercaptalbumin, PSH, and nonmercaptalbumin, P, using a DEAE-cellulose column. The heterogeneous P fractions contained monomer, dimers (which were mostly PSSP formed by oxidation of the sulfhydryl groups), and higher polymers apparently linked by disulfide bonds. Reduction of the monomer with mercaptoethanol yielded a product which contained 1.07 SH/mole of BSA. This would indicate that the monomer, which contained 0.7 SH, contained a small amount of disulfide which is reduced by the mercaptoethanol.

Apparently, only the mixed disulfide in the native albumin is reducible under mild conditions. In the present paper two procedures are presented for the reduction of the mixed disulfide and possibly of PSSP with sodium sulfite under specified conditions. In a previous paper (Kolthoff *et al.*, 1960) it was found that all the disulfide groups in BSA, denatured in 8 M urea, react in this medium with sulfite at a pH near 6. After the reaction was complete the mixture was acidified to pH 2 and the sulfhydryl formed was titrated amperometrically at this pH. We have used a similar method for the determination of the sum of sulfhydryl originally present in the BSA plus the trace of "reactive disulfide" in undenatured albumin. In order to get exact results it was necessary to make a detailed study of the titration of sulfhydryl at pH 2 in the presence of sulfur dioxide. The results are found in the Experimental Section.

A much simpler procedure is to work in ammonia buffer, add sulfite, and titrate the sum of original sulfhydryl and sulfhydryl formed in the reaction and during the titration with a suitable reagent. The reaction with sulfite of disulfide groups in BSA in 8 M urea is highly incomplete at pH 9 (Kolthoff *et al.*, 1959). However, all the disulfide can be titrated because of a continuous shift to the right of the reaction



(2)

It is shown in the present paper that the simple procedure 2 in native albumin at pH 9 yields the same results as the involved procedure in which the titration is made at pH 2.

Experimental Section

Protein Samples. Three commercial lots, A-69805, T-68308, and V-68802, purchased from the Armour Co., have been used. The mole fractions of sulfhydryl in these samples were 0.80, 0.68, and 0.69, respectively.

Chemicals. The same chemicals and methods of purification were used as in previous papers (Kolthoff *et al.*, 1959, 1960, 1965).

Titration Cell (Kolthoff *et al.*, 1965). A beaker of 150-ml capacity, covered with a tight-fitting rubber stopper provided with holes for the electrode, buret, nitrogen inlet and outlet tubes, and the salt bridge, served as titration vessel. The salt bridge was filled with a liquefied gel obtained by dissolving 3.5 g of agar in 100 ml of 3 M potassium nitrate at higher temperature and cooling.

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[1] Abbreviations used in this work: BSA, bovine serum albumin; PSH, mercaptalbumin; PSSR, mixed disulfide; P, nonmercaptalbumin, EMC, ethylmercuric chloride.

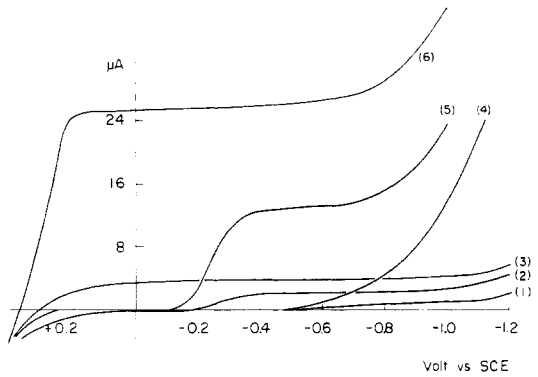

FIGURE 1: Voltammograms of EMC and $HgCl_2$ in 0.01 M $HClO_4$ at rotated mercury wire electrode (RMWE) and rotated dropping mercury electrode (RDME). (1) Blank, 0.005% gelatin, RDME; (2) 2×10^{-5} M in EMC, 0.005% gelatin, RDME; (3) 2×10^{-5} M in $HgCl_2$, 0.005% gelatin, RDME; (4) blank, as (1), RMWE; (5) 2×10^{-5} M in EMC, RMWE; (6) 2×10^{-5} M in $HgCl_2$, RMWE.

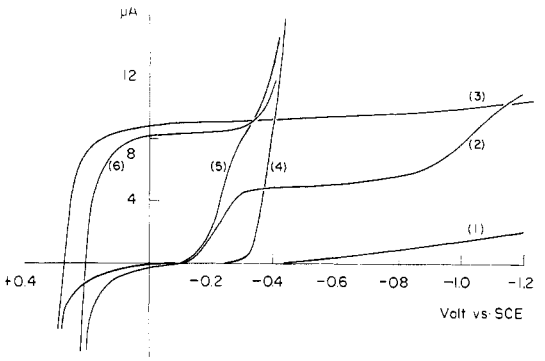

FIGURE 2: Voltammograms of EMC and $HgCl_2$ in 0.5% BSA at pH 2 with and without Na_2SO_3 at the rotated dropping mercury electrode (RDME). All solutions free of oxygen. (1) Blank, 0.5% BSA, 0.01 M $HClO_4$; (2) as (1), but also 1.08×10^{-4} M in EMC; (3) as (1), but also 1.08×10^{-4} M in $HgCl_2$; (4) blank, 0.5% BSA, 0.02 M Na_2SO_3, pH 2.1; (5) as (4), but also 1.64×10^{-4} M in EMC; (6) as (4), but also 1.08×10^{-4} M in $HgCl_2$.

A saturated calomel electrode served as reference electrode.

In current-voltage curve measurements, a stream of nitrogen was first passed through the solution and then over the surface of the solution. In amperometric titrations the gas was passed through the solution continuously.

Titration. All solutions were titrated with exclusion of air. Titrant was added in increments of 0.005 ml from a Manostat ultramicroburet with 0.0002-ml divisions. Unless otherwise stated, the time interval between addition of an increment of titrant and measurement was 1 min.

Electrodes. These have been described in a previous publication (Kolthoff *et al.*, 1965). The rotated dropping mercury electrode had a drop time of 3.1 sec at open circuit and the speed of rotation used in the present work was 180 rpm. The rotated mercury wire electrode consisted of a mercury-coated platinum wire of the loop type. It had a diameter of 0.6 mm and a length of 15 mm and was rotated at a speed of 600 rpm.

Results

Voltammograms at pH 2 at 25°. Voltammograms were run in order to find the potential range at which amperometric titrations should be carried out. Voltammograms of ethylmercuric chloride (EMC) and mercuric chloride were run in 0.01 M perchloric acid at the rotated dropping mercury electrode and the rotated mercury wire electrode. Results are presented in Figure 1. It is seen that the rotated mercury wire electrode used is some eight times more sensitive than the rotated dropping mercury electrode. Figure 2 presents voltammograms at the rotated dropping mercury electrode of EMC and $HgCl_2$ in 0.5% BSA in the absence and presence of sulfite (sulfur dioxide). All solutions were adjusted to pH 2 with perchloric acid and were air-free. Comparing the curves in Figures 1 and 2 reveals that BSA does not affect the general characteristics of the voltammograms and that the mercury mercaptide of the protein is not reducible. The diffusion currents in Figure 2 of EMC and $HgCl_2$ are smaller than corresponds to the added concentrations because of reaction with sulfhydryl in BSA. From the difference we calculate a sulfhydryl content of 0.68 SH, the same as found by direct amperometric titration at pH 8 to 9 or pH 2 (lot T-68308). The amperometric titration in the absence of sulfur dioxide with $HgCl_2$ at pH 2 is carried out at $E_{applied}$ of 0 to −0.1 v, and with EMC at E_{appl} of −0.4 v. The rotated mercury wire electrode is preferred in these titrations over the rotated dropping mercury electrode. With the latter the excess reagent line must be determined very quickly, and only the first part yields a straight line. The reason is that the mercury droplets which collect at the bottom of the cell react with mercuric chloride with the formation of calomel. Fortunately the presence of 0.01 M or more concentrated sulfur dioxide prevents, or at least retards, this reaction, apparently because of formation of a complex sulfitomercurate compound. In the presence of 0.01 to 0.02 M sulfur dioxide the titration with $HgCl_2$ can be carried out at E_{appl} between 0 and −0.3 v; at −0.35 v the sulfur dioxide yields a reduction current (see curves 4, 5, and 6, Figure 2). From curve 5, Figure 2, it is seen that the reduction of SO_2 at the rotated dropping mercury electrode starts just as the potential where the limiting current of EMC is attained; therefore the titration at the rotated dropping mercury electrode in the presence of sulfur dioxide with EMC must be carried out at −0.35 v (*vs.* saturated calomel electrode), a variation of a few hundredths of a volt

TABLE I: Potential in the Amperometric Titration of SH in BSA in Absence and Presence of 0.02 M Sulfite at pH 2.

Indicator Electrode	Titrant	Sulfite	Suitable Potential (*vs.* SCE[c])
RDME[a]	0.05 M $HgCl_2$	Absent	0.00 to −0.30
RDME	0.05 M $HgCl_2$	Present	0.00 to −0.25
RDME	0.05 M EMC	Absent	−0.35 to −0.50
RDME	0.05 M EMC	Present	−0.30 to −0.35
RMWE[b]	0.01 M $HgCl_2$	Absent	+0.10 to −0.40
RMWE	0.01 M $HgCl_2$	Present	−0.10 to −0.25
RMWE	0.01 M EMC	Absent	−0.40 to −0.50
RMWE	0.01 M EMC	Present	−0.30

[a] Rotated dropping mercury electrode, 180 rpm. [b] Rotated mercury wire electrode, 600 rpm. [c] Saturated calomel electrode.

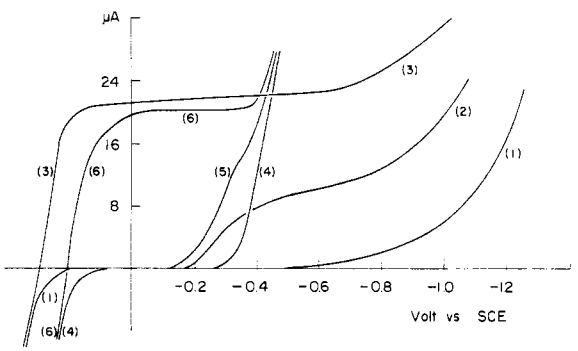

FIGURE 3: Voltammograms of EMC and $HgCl_2$ in 0.1% BSA at pH 2 in the presence and absence of Na_2SO_3 at the rotated mercury wire electrode (RMWE). (1) Blank, 0.1% BSA, 0.01 M $HClO_4$; (2) as (1), but also 2.96×10^{-5} M in EMC; (3) as (1), but also 2.96×10^{-5} M in $HgCl_2$; (4) blank, 0.1% BSA, 0.02 M Na_2SO_3, pH 2.1; (5) as (4), EMC, 2.96×10^{-5} M; (6) as (4), $HgCl_2$, 2.96×10^{-5} M.

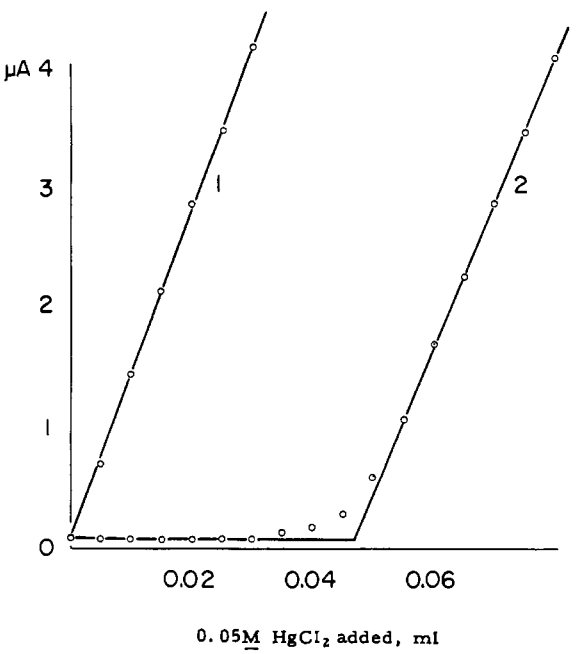

FIGURE 4: Amperometric titration of SH in BSA with 0.05 M $HgCl_2$ at rotated dropping mercury electrode (RDME). (1) Blank, 0.02 M Na_2SO_3, pH 2.2, at −0.1 v, 0.005% gelatin; (2) 0.5% in BSA, 0.02 M Na_2SO_3, pH 2.25, at −0.1 v.

being permissible. Both with $HgCl_2$ and EMC the excess reagent lines are straight. The titration with $HgCl_2$ is much preferred. However, in the present work many titrations have been carried out at −0.35 v with EMC. The reason is that mercuric chloride is bifunctional; it reacts with SH in BSA in a mole ratio of 1:1, but with SH liberated from disulfide (Kolthoff, *et al.*, 1958) in the ratio 1:2. Actually, in the present paper it is shown that $HgCl_2$ reacts with SH liberated by sulfite from "reactive disulfide" in the 1:2 ratio.

From Figure 3 it appears that the rotated mercury wire electrode is very suitable for SH titrations at pH 2 in the absence of sulfur dioxide (curves 2, 3). However, in the presence of sulfur dioxide with EMC the diffusion current of the excess reagent can hardly be measured. From curve 5 in Figure 3 it is seen that at −0.35 v the diffusion current of EMC is virtually attained, but that reduction of sulfur dioxide has started already. This yields a relatively large residual current. We have carried out titrations with EMC at the rotated mercury wire electrode in the presence of sulfur dioxide at a potential of −0.30 v. However, the rotated dropping mercury electrode is preferred in the presence of sulfur dioxide. Table I lists suitable applied potentials in titrations with $HgCl_2$ and EMC at pH 2. Some typical sulfhydryl titrations at the rotated dropping mercury electrode with $HgCl_2$ and EMC at pH 2 in the presence of sulfur dioxide are presented

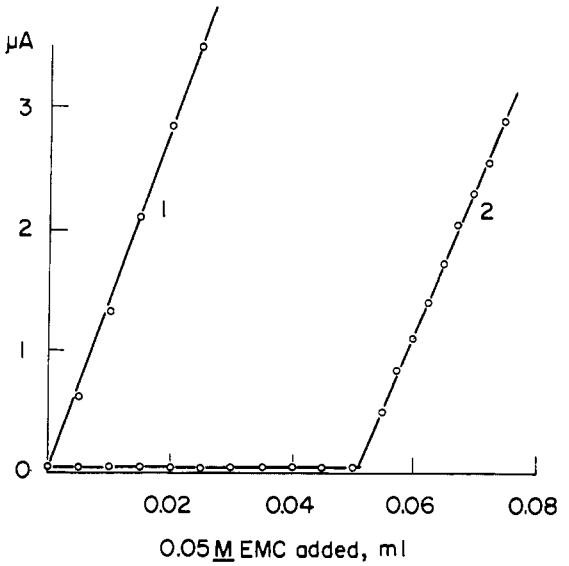

FIGURE 5: Amperometric titration of SH in BSA with 0.05 M EMC at rotated dropping mercury electrode (RDME). (1) Blank, 0.02 M Na_2SO_3, pH 2.1, at −0.30 v, 0.005% gelatin; (2) 0.5% BSA, 0.02 M Na_2SO_3, pH 2.3, at −0.30 v, 50 ml.

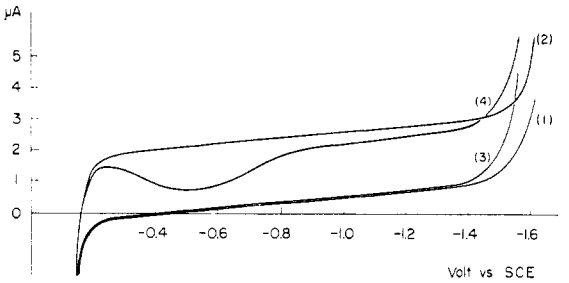

FIGURE 6: Voltammograms of $HgCl_2$ in 0.1 M ammonia buffer, pH 9.2, at rotated dropping mercury electrode (RDME). (1) Blank, 0.1 M ammonia buffer, 0.05 M Na_2SO_3, 0.005% gelatin; (2) as (1), but 2×10^{-5} M in $HgCl_2$; (3) blank, 0.1 M ammonia buffer, 0.05 M Na_2SO_3, 0.2% BSA (2.9×10^{-5} M); (4) as (3), but 4×10^{-5} M in $HgCl_2$.

in Figures 4 and 5. It is of interest to add that sulfur dioxide decreases the diffusion current of mercuric chloride. The decrease is much greater at the rotated mercury wire electrode than at the rotated dropping mercury electrode. For example, in the presence of 0.02 M sulfur dioxide (pH 2) the diffusion current at the rotated dropping mercury electrode was decreased by 10% and in 0.1 M sulfur dioxide by 15%. At the rotated mercury wire electrode the decrease was 30 and 70%, respectively. Mercury(II) forms complexes with sulfur dioxide which, apparently, are reduced more rapidly at the rotated dropping mercury electrode than at the rotated mercury wire electrode.

A special study was made of the effect of chloride on the sulfhydryl titration at pH 2. At the rotated dropping mercury electrode or rotated mercury wire electrode 0.1 M chloride does not interfere in titrations with mercuric chloride, either in the presence or absence of sulfur dioxide. However, chloride does interfere in titrations with EMC in the presence of sulfur dioxide. EMC is very slightly dissociated. Excess chloride suppresses the dissociation. Consequently, the $C_2H_5Hg^+$ ion concentration is decreased and chloride shifts the wave to more negative potentials. For example, in the absence of added chloride, with 0.01 M and 0.1 M chloride $E_{1/2}$ of EMC was −0.21, −0.37, and −0.43 v, respectively. In the presence of added chloride the waves overlap with the reduction current of sulfur dioxide, and no suitable potential is found at which the titration can be carried out. Traces of chloride up to 0.002 M do not interfere. Oxygen interferes in titrations with EMC. In titrations with mercuric chloride it is possible to select a potential at which fairly satisfactory results are obtained. However, it is recommended to remove oxygen, and this has been done in the subsequent work at pH 2.

Sulfite distorts voltammograms of mercuric chloride at pH 9 at the rotated dropping mercury electrode and rotated mercury wire electrode. From Figure 6 it is seen that the diffusion current of $HgCl_2$ at the rotated dropping mercury electrode is approached at −0.2 v; however, with increasing negative potential the current goes through a minimum to attain the value of the diffusion current at −0.8 v (see Figure 6, curve 4; uncorrected for i_r). At the rotated mercury wire electrode the distortion is so bad (Figure 7) that no diffusion current is attained.

Procedures for Reactive Disulfide

Procedure 1. A solution of 0.25 g of BSA in 20 ml of deaerated conductivity water was made in a 25 ml volumetric flask, 1.0-ml of air-free 1 M perchloric acid and 1.25 ml of 1 M sodium sulfite were added, and the mixture was diluted to the mark. A very small drop of octanol was added to minimize foaming, and nitrogen was bubbled through for 10 min. The pH of the mixture was 6.2. After standing for 1 hr 10 ml of the mixture was added by pipet to 40 ml of deaerated 0.03 M $HClO_4$ in the titration vessel to give a solution of pH 2. After bubbling through nitrogen for 10 min, the total sulfhydryl was titrated with 0.02 M mercuric chloride at −0.1 v at the rotated mercury wire electrode or with 0.05 M EMC at −0.30 to −0.35 v at the rotated dropping mercury electrode. The "reactive disulfide" was found by subtracting the sulfhydryl content of the BSA from total sulfhydryl found in the titration.

Procedure 2. In the titration cell 0.1–0.2 g of BSA was dissolved in 47.5 ml of 0.1 M ammonia–ammonium nitrate buffer, pH 9.2. After dissolution, 2.5 ml of 1 M sodium sulfite was added, and the sulfhydryl was then titrated with the different titrants. The titration with silver nitrate was carried out at −0.3 to −0.6 v (*vs.* saturated calomel electrode) at the rotated platinum

TABLE II: Reactive Disulfide Plus Sulfhydryl in Moles/Mole in Native BSA.

Lot of BSA	SH	Procedure 1 EMC[a]	Procedure 1 $HgCl_2$[a,b]	Procedure 2 EMC[a]	Procedure 2 $HgCl_2$,[a,b]
A-69805	0.80	1.00; 1.00	1.03; 0.99	1.00; 0.99; 0.99	1.03; 1.03
T-68308	0.68	0.92; 0.89	0.90; 0.90	0.91; 0.92	0.94; 0.96
V-69012	0.69	0.91; 0.92	1.02; 1.03	0.92; 0.93	1.00; 0.99

[a] As titrants. [b] Calculated on basis that $HgCl_2$ reacts with PSH in molar ratio 1 : 1, but in ratio 1 : 2 with SH liberated by sulfite.

wire electrode. The titration with EMC at the rotated dropping mercury electrode was carried out at −0.8 v and with mercuric chloride at −0.8 to −1.2 v. For reasons given below the current on the excess reagent line was measured within 1 min after addition of each increment of titrant. Again, the molar reaction ratio of mercuric chloride to sulfhydryl liberated by sulfite was found to be 1 : 2. The objection may be raised that procedure 2 yields an arbitrary value, since reaction 2 is an equilibrium reaction and the equilibrium may be displaced continuously on increment addition of titrant. If this happened, higher and less reproducible results than in procedure 1 would be found. As is evident from the results in Table II, this is not the case. The following experiments also show that none of the 17 disulfide groups/mole of BSA reacts in procedure 2. The sum of reactive S–S and SH in lot A-69805 (SH content 0.80) was titrated with EMC by procedure 2 and found to be 1.00/mole of protein. Another experiment was made in which an amount of EMC corresponding to 1.0 S–S plus SH was added. The solution was then allowed to stand for 10 min to 3 hr and the excess reagent line determined. The sum of S–S + SH remained one, even after 3 hr of standing. It is quite evident then that none of the "normal" disulfide reacts with sulfite under the conditions of procedure 2. The 17 disulfide groups in BSA can react quantitatively with sulfite at pH 9 (or less) when an excess of EMC, mercuric chloride, or silver nitrate is added and the mixture allowed to stand for about 20 hr. The reaction is slow, but may occur to some extent when the excess reagent line in procedure 2 is determined too slowly. When current was measured within 2 min after addition of each increment of excess of EMC the same results were obtained as in procedure 1. When the interval was 3 min the results were 4% high, and they became higher with longer time intervals. Also, when 20% or more EMC in excess was added to a reaction mixture and this was allowed to stand for 10 min or more, increasing amounts of EMC were used up with increasing time of standing.

Table II summarizes all the results obtained using both procedures with 3 lots of BSA. The sulfhydryl content of the lot of BSA used is mentioned in the second column. Reactive disulfide is not reported; it is equal to the sum of S–S + SH − SH.

Silver nitrate was used in procedure 2 with only one lot of BSA. The results are somewhat lower than those obtained with EMC and mercuric chloride. The residual current increased continuously before the end point, the latter being not sharply defined. Silver nitrate as titrant is not recommended for the present purpose.

It may be concluded that the sum of reactive disulfide and sulfhydryl in BSA is equal to one/mole of albumin. To substantiate this conclusion we requested Dr. R. W. Hartley of the National Cancer Institute to send us samples of the fractions of BSA which he and his as-

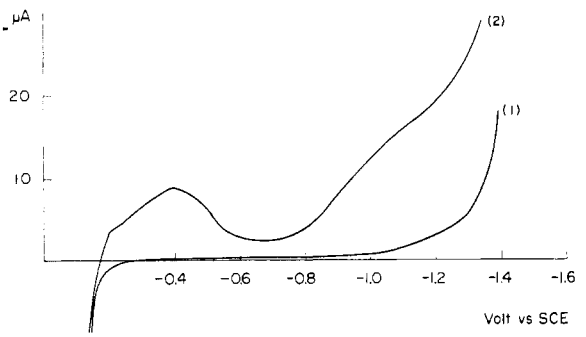

FIGURE 7: Voltammograms of $HgCl_2$ in ammonia buffer at rotated mercury wire electrode (RMWE). (1) Blank, 0.1 M ammonia buffer, 0.05 M Na_2SO_3, 2.9 × 10^{-5} M BSA; (2) as (1), but 4 × 10^{-5} M in $HgCl_2$.

TABLE III: Sum of SH + Reactive Disulfide in Fractions of BSA Separated by Hartley *et al.* (1962).

Fraction	SH (per mole of protein)	SH + SS (per mole of protein)
1	0.28	0.81
2	0.51	0.95
3	0.93	1.00
4	0.28	0.72
5	0.63	1.02
6	0.95	0.99

sociates prepared by frontal and/or sectioned column chromatography in DEAE-cellulose. He kindly complied with our request. We mixed those fractions which had a similar sulfhydryl content and analyzed these for the water, sulfhydryl, and reactive disulfide. The results are presented in Table III. Dr. Hartley stated to us that none of the fractions contained more than 1–2% dimer. Fractions 1 and 4 in Table III are composed mainly of nonmercaptalbumin. In these fractions the sum of sulfhydryl and reactive disulfide was 0.81 and 0.72, respectively. In fractions 2 and 5, which were low in SH, this sum was close to 1. Fractions 3 and 6, which were virtually composed of pure mercaptalbumin, practically did not contain reactive disulfide. Thus, in 4 of the 6 fractions SH + S–S was 1. It also may be concluded that pure mercaptalbumin does not contain "reactive disulfide".

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Synthesis of Angiotensins by the Solid-Phase Method*

Garland R. Marshall and R. B. Merrifield

ABSTRACT: The new method of solid-phase synthesis was applied to the preparation of isoleucine^5-angiotensin II. These syntheses started with *t*-butyloxycarbonylphenylalanylcopolystyrene–divinylbenzene, ended with chromatographically pure octapeptide, and gave an over-all yield of 56%. The synthetic angiotensin possessed full oxytocic activity. Since the rearrangement of the α-aspartyl bond to the β-aspartyl bond had been reported under conditions similar to those used in the synthesis, it was then ascertained by a variety of methods that this rearrangement did not occur in solid-phase synthesis. The analogs, asparagine^1-isoleucine^5-angiotensin II and β-aspartic^1-isoleucine^5-angiotensin II, were also synthesized.

The solid-phase method has recently been introduced to speed, simplify, and automate the synthesis of peptides and, ultimately, proteins (Merrifield, 1962, 1965). Both bradykinin and methionyllysylbradykinin have been successfully synthesized in this way (Merrifield, 1963, 1964a,b). This report on the syntheses of the biologically active octapeptide, isoleucine^5-angiotensin II, and two of its analogs presents further evidence in support of the benefits and applicability of the solid-phase method.

Page and Helmer (1939) and Braun-Menendez *et al.* (1939) announced simultaneously the discovery of a pressor substance resulting from the action of the renal proteolytic enzyme, renin, on plasma. It was later shown that the product of the reaction of renin with plasma is an inactive decapeptide (angiotensin I) which is further degraded by a plasma enzyme to the biologically active octapeptide, angiotensin II (Skeggs *et al.*, 1954, 1956a). The structure of the active peptide from the horse (Skeggs *et al.*, 1956b) has been confirmed by synthesis (Schwarz *et al.*, 1957; Schwyzer *et al.*, 1957; Arakawa and Bumpus, 1961). It is designated isoleucine^5-angiotensin II in order to distinguish it from the corresponding peptide of bovine origin which contains valine in position five.

The synthesis of isoleucine^5-angiotensin II by the solid-phase method was undertaken for two reasons. First, it was of interest as a further test of the applicability of this new method of peptide synthesis. Since angiotensin contained four amino acids, aspartic acid, histidine, isoleucine, and tyrosine, which had not previously been introduced into peptides in this way, the synthesis provided additional evidence for the general applicability of solid-phase synthesis. Second, it was expected to provide a simplified synthetic route to this important compound and various derivatives. The synthesis followed the basic concept of solid-phase peptide synthesis as outlined previously (Merrifield, 1964a), but differed in that the coupling steps were carried out in methylene chloride where possible. The steps involving

*The Rockefeller University, New York, N. Y. 10021. This study was made in partial fulfillment of the requirement for the Ph.D. degree by G. R. Marshall. An abstract of this work was presented at the 49th Annual Meeting of the Federation of American Societies for Experimental Biology, April 1965 (Marshall and Merrifield, 1965). This is paper number V of this series.